

# Randomized, placebo-controlled trial to assess the safety and immunogenicity of an adenovirus type 35-based circumsporozoite malaria vaccine in healthy adults

C Buddy Creech<sup>1,\*</sup>, Cornelia L Dekker<sup>2</sup>, Dora Ho<sup>2</sup>, Shanda Phillips<sup>1</sup>, Sally Mackey<sup>2</sup>, Cristina Murray-Krezan<sup>3</sup>, Maria Grazia Pau<sup>4</sup>, Jenny Hendriks<sup>4</sup>, Valerie Brown<sup>5</sup>, Leonard G Dally<sup>5</sup>, Isabella Versteeg<sup>4</sup>, and Kathryn M Edwards<sup>1</sup>

<sup>1</sup>Vanderbilt Vaccine Research Program; Department of Pediatrics; Vanderbilt University School of Medicine; Nashville, TN USA; <sup>2</sup>Stanford-LPCH Vaccine Program, Department of Pediatrics—Infectious Diseases; Stanford University School of Medicine; Stanford, CA USA; <sup>3</sup>Division of Epidemiology, Biostatistics and Preventive Medicine; Department of Internal Medicine; University of New Mexico Health Sciences Center; Albuquerque, NM USA; <sup>4</sup>Crucell; Leiden, The Netherlands; <sup>5</sup>EMMES Corporation; Rockville, MD USA

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Malaria results in over 650 000 deaths each year; thus, there is an urgent need for an effective vaccine. Pre-clinical studies and recently reported human trials suggest that pre-erythrocytic stage vaccines can provide protection against infection. A Phase 1, randomized, placebo-controlled, dose-escalation study was conducted with a vaccine composed of a replication-deficient adenovirus-35 backbone with *P. falciparum* circumsporozoite (CS) surface antigen (Ad35.CS.01). Healthy adult subjects received three doses of 10<sup>8</sup>, 10<sup>9</sup>, 10<sup>10</sup>, or 10<sup>11</sup> vp/mL Ad35.CS.01 vaccine or saline placebo intramuscularly at 0, 1, and 6-mo intervals. Adverse events were assessed and anti-CS antibody responses were determined by ELISA. Seventy-two individuals were enrolled, with age, gender, and ethnicity similar across each study arm. While the vaccine was generally well tolerated, adverse events were more frequent in the highest dose groups (10<sup>10</sup> and 10<sup>11</sup> vp/mL). More robust humoral responses were also noted at the highest doses, with 73% developing a positive ELISA response after the three dose series of 10<sup>11</sup> vp/mL. The Ad35.CS.01 vaccine was most immunogenic at the highest dosages (10<sup>10</sup> and 10<sup>11</sup> vp/mL). Reactogenicity findings were more common after the 10<sup>11</sup> vp/mL dose, although most were mild or moderate in nature and resolved without therapy.

## Introduction

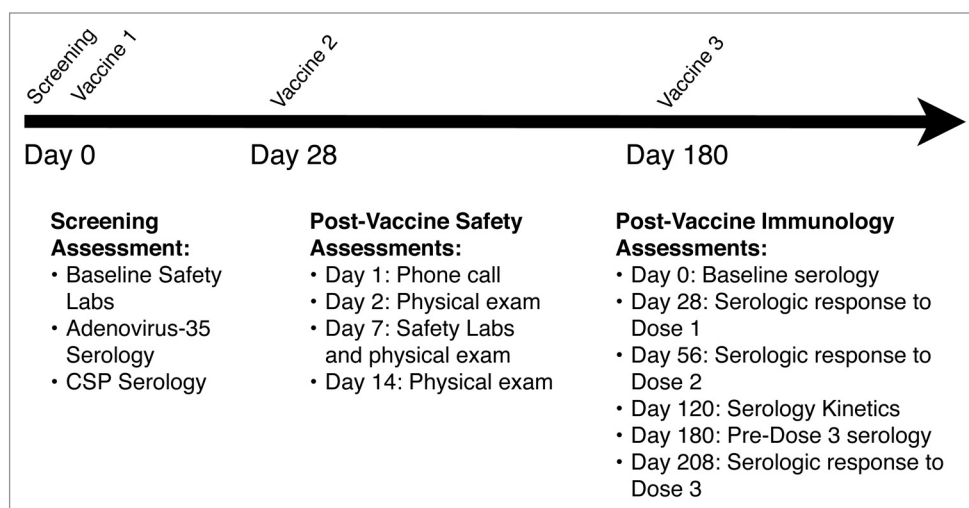
Malaria is one of the most prevalent global infectious diseases with more than 200 million infections and over 650 000 deaths annually.<sup>1–3</sup> As a result of drug-resistant parasites and insecticide-resistant mosquitoes, malaria continues to pose a public health threat.<sup>1</sup> This threat is worsened by environmental and climatic changes, civil disturbances, and increased mobility of the human population.

Malaria is caused by mosquito-borne hematoprotzoan parasites belonging to the genus *Plasmodium*.<sup>4</sup> Five species of *Plasmodium* protozoa (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) are responsible for human disease, with *P. falciparum* accounting for the majority.<sup>4</sup> The parasite life cycle has multiple stages, with each stage inducing specific immune responses against specific expressed antigens that may modify disease risk. Initially, infected mosquitoes inject sporozoite stage parasites into the mammalian bloodstream, where the parasites are exposed to host antibodies (in those who are

malaria-experienced) primarily directed to the circumsporozoite (CS) protein, a major surface component of the sporozoite. Field studies have correlated antibodies to CS protein and other pre-erythrocytic antigens with protection from *P. falciparum* infection.<sup>5–7</sup> Additionally, the recently reported modest efficacy of a CS-based malaria vaccine candidate (RTS, S) suggests that CS vaccines afford some protection in malaria hyper- and holoendemic areas.<sup>8–15</sup> However, a more highly protective malaria vaccine is needed.

Immunity to malaria is decidedly complicated, with a combination of humoral and cellular immunity working together to reduce mortality and morbidity and to decrease the overall parasite burden in the human host.<sup>1</sup> The vaccine candidate Ad35.CS.01 has a codon optimized nucleotide sequence representing the *P. falciparum* circumsporozoite (CS) surface antigen inserted into a replication deficient adenovirus-35 (Ad35) vector.<sup>16–18</sup> As a pre-erythrocytic vaccine candidate, Ad35.CS.01 would be expected to work by increasing neutralizing antibodies that could inhibit *Plasmodia* sporozoites from entering the

\*Correspondence to: C Buddy Creech; Email: buddy.creech@vanderbilt.edu  
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**Figure 1.** Schedule of events and outcome measures. Baseline screening assessments included complete blood counts; serum chemistries; hepatic enzymes; urinalysis; serologies for HIV, HCV, and HBV; and pregnancy testing. During post-vaccine safety visits, each subject's memory aid was reviewed for adverse events. The primary outcome measure was the frequency and severity of local and systemic adverse events. Secondary outcome measures included anti-CS antibody titers (ELISA) and neutralizing antibody titers to adenovirus type 35.

hepatocyte. In addition, a successful vaccine candidate might be able to destroy infected hepatocytes, harnessing CD4<sup>+</sup>, CD8<sup>+</sup>, natural killer T, and  $\gamma\delta$ T-cells to inhibit intrahepatic parasites. Preclinical data are certainly encouraging, given the high CSP-specific cellular and humoral responses observed in mice vaccinated with a combination of Ad35 and Ad5 vectored CSP vaccines and a significant reduction in hepatic infection upon malaria challenge.<sup>16,18–20</sup>

Adenoviral vectors are attractive for vaccines because the genome is well characterized, easy to manipulate, and capable of being rendered replication-defective. Adenovirus-vectored constructs are also exciting vaccine candidates due to their ability to induce potent T-cell and B-cell memory responses<sup>21</sup> and to boost the response to other CS antigen vaccines. Several adenovirus-vectored CS constructs exist, each with potential advantages.<sup>22,23</sup> Recombinant Ad5 expressing *P. yoelii* derived CS protein (Ad5PyCS) provided potent CD8<sup>+</sup> T-cell responses and protection against subsequent challenge in mice models.<sup>24</sup> Similarly, Ad26 and Ad35 vectored vaccines are known to stimulate both humoral and cellular responses,<sup>25</sup> though the protection afforded by these constructs alone is uncertain. Using a prime-boost strategy, Chuang et al. tested a DNA prime/adenovirus boost in a Phase I malaria challenge study. In this strategy, Ad5-CS and Ad5-AMA1 (apical membrane antigen-1) were given after DNA plasmid prime. This regimen provided modest protection against controlled challenge, but not in those with high pre-existing antibodies to Ad5.<sup>26–28</sup> A different prime-boost strategy of Ad35.CS followed by RTS, S in primates resulted in cell-mediated immune responses that were both higher and more durable than those seen following either vaccine alone.<sup>17</sup> While CD8<sup>+</sup>, IFN $\gamma$  secreting T-cells in pre-clinical animal models have been implicated in protection against malaria challenge,<sup>29</sup> primate studies show a predominance of CD4<sup>+</sup> T cells after prime-boost vaccination with Ad35.CS followed by RTS,

S. The reported success of 30–60% of RTS, S in field efficacy studies<sup>11,30</sup> leaves room for strategies to further increase efficacy.

The primary objective of this trial was to assess the safety of the Ad35.CS.01 malaria vaccine among healthy subjects. A secondary objective was to assess the immunogenicity of the vaccine, with assessment of cellular immune responses an exploratory endpoint.

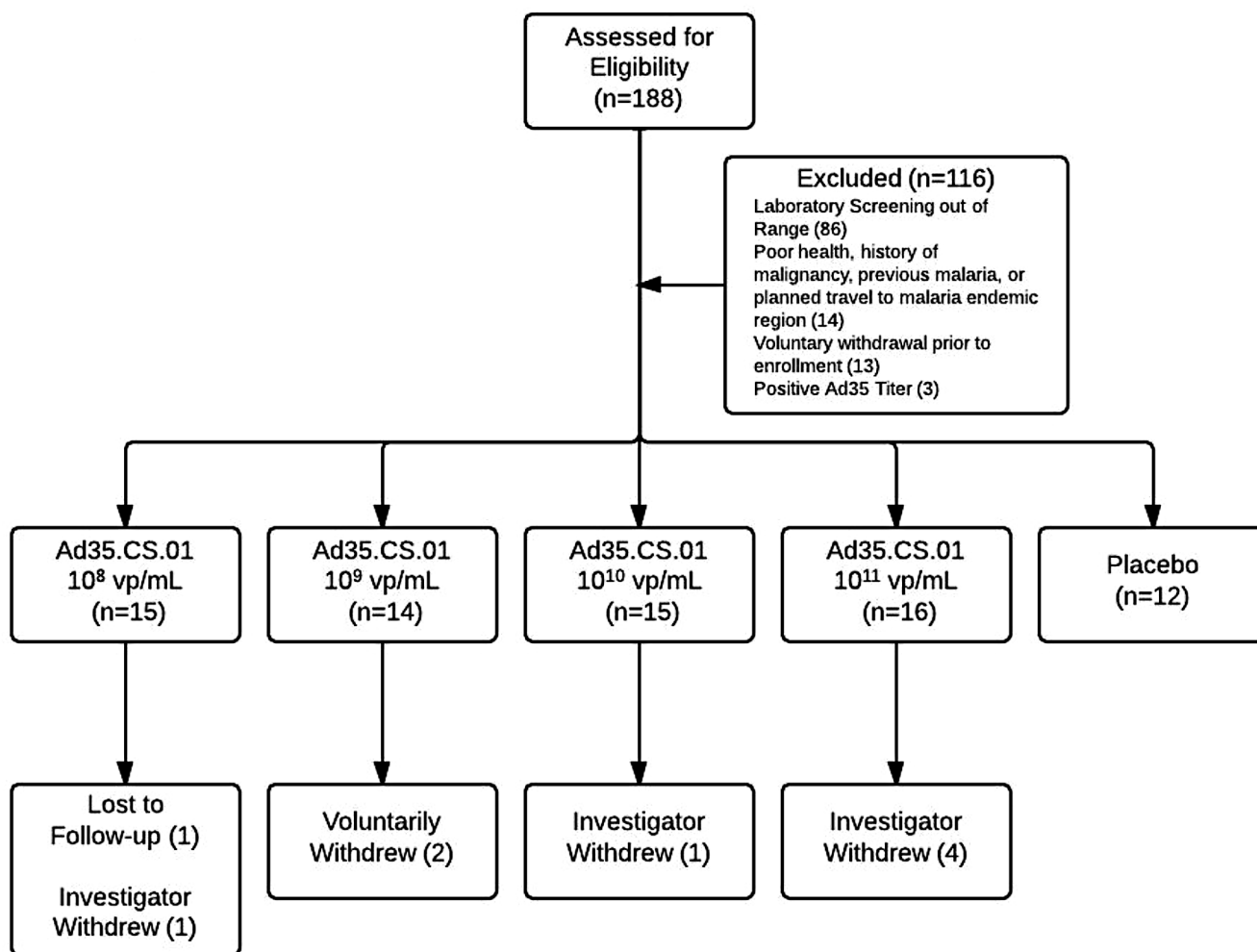
## Results

### Study completion

Seventy-two subjects were recruited into the study with an equal number of each gender and a mean age of 28 y (Fig. 1). All placebo recipients completed the three dose series. In the 10<sup>8</sup> viral particles (vp)/mL group, two subjects discontinued the trial, both after the 2nd dose: one was lost to follow up, and one was terminated due to a dispensing error. In the 10<sup>9</sup> vp/mL group, two subjects withdrew their consent for personal reasons unrelated to the trial and completed only the first dose. In the 10<sup>10</sup> vp/mL group, 1 subject was withdrawn by the investigators due to noncompliance (participation in another clinical trial). Finally, in the 10<sup>11</sup> vp/mL group, four subjects were discontinued due to adverse events that are discussed below. All adverse events in these subjects resolved without treatment or sequelae.

### Solicited local reactions

Local injection site pain was generally mild in nature but increased in frequency with increasing dosages. As illustrated in Figure 2A, while the reactions seen after the dosage of 10<sup>8</sup> vp/mL were not distinguishable from placebo, the frequency of subjects reporting pain increased with increasing dosages. The 10<sup>11</sup> vp/mL dosage was associated with more reports moderate pain, particularly after dose 3. Pain was reported only on the day of injection, being either resolved or decreased to mild pain the



**Figure 2.** CONSORT Flow Diagram of Study Participants. In total, 62/72 subjects completed all vaccinations and follow-up visits. Reasons for voluntary withdrawal include loss to follow-up, concern about risks and study procedures, and insufficient time to complete study visits. Reasons for investigator withdrawal include non-compliance due to participation in another trial (1), dosing error (1), and adverse reactions to vaccine (4). Adverse events leading to withdrawal occurred exclusively in the Ad35.CS.01 10<sup>11</sup> vp/mL dosage group.

following day. No subject reported severe pain. Mild erythema was common in both placebo and study product recipients.

#### Solicited systemic reactions

Fever was uncommon in placebo recipients and in the lower dose cohorts (Fig. 2B), but more than 20% of subjects in the 10<sup>11</sup> vp/mL dose cohort experienced fever (>100.0 °F). The average duration of reported fever was ≤1 d. The numbers of subjects experiencing headache, malaise, myalgia, and nausea are presented in Figure 2C–F, respectively. All systemic symptoms other than headache were more common among the highest relative to lower dose groups ( $p < 0.05$  for each).

Table 1 summarizes the number and proportion of subjects experiencing any solicited adverse event (AE) by maximal severity. Systemic reactogenicity following the first administration of the three lower doses was similar to that observed after receipt of 10<sup>10</sup> vp/mL. However, subjects in the 10<sup>11</sup> vp/mL dose group were more likely to experience reactions when compared with placebo

(82% of subjects vs. 25%;  $p = 0.01$ ). 13% of subjects in the highest dose group experienced events that were rated as severe based on interruption of activities of daily living. Tolerability of the 10<sup>10</sup> vp/mL dose was only slightly less favorable than placebo with 41% of subjects experiencing solicited systemic reactions including one severe headache. Following the second and third vaccinations, the overall frequency of systemic reactions was similar across the three lowest dosages relative to placebo; however 10<sup>11</sup> vp/mL induced more frequent reactions of any grade ( $p = 0.06$ ) and of moderate or severe grade ( $p = 0.01$ ). In total, more than 90% of subjects receiving the 10<sup>11</sup> dose experienced at least 1 solicited systemic AE.

#### Overview of serious adverse events and laboratory adverse events

One serious adverse event (SAE), a small bowel obstruction requiring hospitalization and surgery that was deemed unrelated to study product, was reported 74 d after the 3rd injection. After final study unblinding, it was learned that the subject had

received placebo. In addition, transient mild increases in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) within 14 d of vaccination were seen in 12 subjects; however, while 8/60 vaccine recipients had elevated ALT, 4/12 of the placebo recipients also had elevated ALT ( $p > 0.10$ , Table S1).

#### Unsolicited adverse events

In total, 241 unsolicited AEs were reported through Day 208; 66 (58 mild; 7 moderate; 1 severe) were considered associated with the study product. The frequencies of subjects experiencing associated AEs in the placebo,  $10^8$ ,  $10^9$ ,  $10^{10}$ , and  $10^{11}$  vp/mL groups were 17%, 14%, 47%, 33%, and 31%, respectively. All events were transient and resolved spontaneously without sequelae. These events are characterized further by dosage cohort below.

##### *$10^8$ vp/mL cohort*

Seven unexpected, unsolicited adverse events were considered related to study product. One subject experienced paresthesias in the vaccinated arm after the 2nd and 3rd doses of the vaccine. The events were not associated with motor weakness or sensory loss, and symptoms resolved within 12 h of vaccine administration. The subject had a history of motor weakness and paresthesias in the right arm following childbirth 13 y earlier. Magnetic resonance imaging (MRI) of the spine obtained 18 mo prior to the study (due to musculoskeletal injury following an automobile accident) showed no anatomic abnormalities or nerve root compressions. One subject had a decreased neutrophil count, and another was reported to have anemia. Each of these resolved without sequelae.

##### *$10^9$ vp/mL cohort*

Twenty events were considered related to study product. Each of these were mild, transient, laboratory abnormalities, including mild elevations of AST/ALT in 4 subjects, hyperglycemia and hypoglycemia (in 3 subjects each), elevated creatinine (1); mild neutropenia (1); and proteinuria (3). All AE's were followed to resolution and none experienced sequelae.

##### *$10^{10}$ vp/mL cohort*

Ten mild and one moderate event were identified as potentially related to the vaccine. One subject reported a pruritic tongue lesion of moderate severity 4 d after vaccination. No other symptoms were noted and the tongue lesion resolved spontaneously after one week. Other AE's included mild eosinophilia (1 subject); pharyngitis and cervical adenopathy in a subject whose viral cultures and adenoviral PCR were negative; an erythematous arm lesion adjacent to the injection site (1); headache (1); herpes simplex infection (1); and proteinuria (2 subjects). One subject developed subtle post-immunization weakness of the immunized arm, noticed during weight lifting. Each of these resolved without intervention.

##### *$10^{11}$ vp/mL cohort*

Subjects in the highest dose group experienced 15 mild, 3 moderate, and 1 severe adverse events judged associated with the vaccine. Four subjects were withdrawn by the investigators due to significant reactogenicity or unsolicited adverse events. The first subject developed diaphoresis, tachycardia, and diarrhea that eventually resulted in syncope on the day following vaccination. This subject, who also developed iron-deficiency anemia, was

later found to suffer from anorexia nervosa, unknown to study team at time of enrollment. The second subject experienced two transient episodes of weakness in the vaccinated arm, associated with transient loss of balance. Another subject experienced grade 3 fever and chills (and was withdrawn from subsequent vaccinations) and a final participant experienced a brachial plexopathy of moderate severity following dose 2. This latter subject described pain and paresthesias in the anterior aspect of the forearm and hand 2 d after vaccination; however, these symptoms resolved spontaneously in the week following vaccination. Each of these events was considered related to study product and resolved without intervention.

#### Immunogenicity (humoral response)

Twenty-eight days following the first vaccination, no appreciable anti-CS antibody levels were identified among the  $10^8$  or  $10^9$  vp/mL dose groups (Table 2), but subjects in the  $10^{10}$  and  $10^{11}$  groups had significantly higher geometric mean antibody titers (GMTs) than placebo ( $p < 0.05$ ). Detectable antibody levels were identified in 6/15 (40%) and 10/14 (71%) of subjects in these respective dose groups after the first vaccine dose. Twenty-eight days after the second vaccination, 7/15 (47%; 95% CI 21–72%) in the  $10^{10}$  and 10/14 (71%; 95% CI 48–95%) in the  $10^{11}$  group had a  $\geq 4$ -fold increase in antibody from pre-vaccination levels. GMTs remained significantly higher among these dose groups relative to placebo ( $p < 0.001$ ). The third vaccination yielded minimal increases in antibody titers relative to the previous dose: 28 d after the third vaccination (Day 208) 4/14 (29%; 95% CI 5–52%) in the  $10^{10}$  and 7/11 (64%; 95% CI 35–92%) in the  $10^{11}$  group had a  $\geq 4$  fold rise in anti-CS antibody levels at Day 208 vs. baseline. One month after the third vaccination, the  $10^{11}$  group demonstrated the highest GMT (61 ELISA units (EU)/mL; 95% CI: 21–176). The frequencies of subjects who had  $\geq 4$ -fold increases in GMT at Day 208 were 7/14 (50%) and 8/11 (73%) in the  $10^{10}$  and  $10^{11}$  vp/mL groups, respectively.

All subjects had undetectable neutralizing antibody titers against Ad35 at the start of vaccination (Fig. 3). In the lowest dosage group of  $10^8$  vp/mL, no positive Ad35 neutralizing antibodies were detected at the end of the three-dose series. In all other groups the number of subjects with detectable antibody titers increased modestly at one month after the 3rd vaccination to 5/13 (38%), 9/14 (64%), and 5/11 (45%) in groups  $10^9$ ,  $10^{10}$ , and  $10^{11}$  vp/mL, respectively.

#### Discussion

This is the first report on the safety and immunogenicity of a novel, adenovirus-vectored malaria vaccine candidate administered to humans. In this study, we demonstrated that an Ad35, CS.01 malaria vaccine targeting the CS antigen was modestly immunogenic in doses ranging from  $10^9$  to  $10^{11}$  vp/mL, and that local and systemic reactogenicity increased in the highest dosage groups. Common local and systemic reactions included injection site pain, erythema, headache, and fever; these symptoms were self-limited and typically mild in nature. Additional adverse events—such as brachial plexopathy, arm weakness, and severe systemic



**Table 1.** Number (%) of subjects with solicited systemic reactions, by maximal severity, after each injection

		Placebo (n = 12)	Ad35.CS.10 <sup>8</sup> (n = 14)	Ad35.CS.10 <sup>9</sup> (n = 15)	Ad35.CS.10 <sup>10</sup> (n = 15)	Ad35.CS.10 <sup>11</sup> (n = 16)
<b>First dose</b>	Mild	1 (8)	3 (21)	2 (13)	4 (27)	6 (38)
	Moderate	2 (17)	2 (14)	0 (0)	1 (7)	5 (31)
	Severe	0 (0)	0 (0)	0 (0)	1 (7)	2 (13)
		<b>n = 12</b>	<b>n = 14</b>	<b>n = 13</b>	<b>n = 15</b>	<b>n = 14</b>
<b>Second dose</b>	Mild	4 (33)	2 (14)	3 (23)	4 (27)	7 (50)
	Moderate	1 (8)	2 (14)	3 (23)	4 (27)	4 (29)
	Severe	0 (0)	1 (7)	0 (0)	0 (0)	1 (7)
		<b>n = 12</b>	<b>n = 12</b>	<b>n = 13</b>	<b>n = 14</b>	<b>n = 12</b>
<b>Third dose</b>	Mild	2 (17)	6 (50)	1 (8)	1 (7)	6 (50)
	Moderate	0 (0)	2 (17)	1 (8)	5 (36)	3 (25)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)
		<b>n = 12</b>	<b>n = 12</b>	<b>n = 13</b>	<b>n = 14</b>	<b>n = 12</b>
<b>Any dose</b>	Mild	5 (42)	5 (36)	4 (27)	4 (27)	4 (25)
	Moderate	2 (17)	4 (29)	4 (27)	6 (40)	7 (44)
	Severe	0 (0)	1 (7)	0 (0)	1 (7)	4 (25)

p < 0.10 for comparison of frequency of events of any grade between Ad35.CS10<sup>11</sup> and placebo. p < 0.05 for comparison of frequency of events of moderate or severe grade between Ad35.CS.01 10<sup>11</sup> and placebo.

reactogenicity—required exclusion from subsequent vaccination in four subjects in the highest dosage cohort although subjects recovered quickly without specific therapy or sequelae. These reactions were self-limited, temporally associated with vaccination/injection, and not due to other established causes. However, their relationship to the study product could not be established in all cases. Doses of at least 10<sup>10</sup> vp/mL were required to generate a 4-fold rise in antibody titers from baseline and only the 10<sup>11</sup> vp/mL dosage generated a sustained humoral response after the 3rd vaccination. Taken together, dosage modifications or heterologous prime-boost strategies will be required for future trials.

One potential disadvantage of an adenoviral vaccine approach is that pre-existing neutralizing antibodies to some adenovirus strains, such as Ad5, can be found in up to 80% of adults<sup>31</sup>; in contrast, antibodies to Ad35 are typically found in <15% of healthy adults globally.<sup>31</sup> Indeed, only four subjects in our study were identified with a detectable Ad35 neutralizing antibody titer in our baseline screening of 91 individuals. The importance of pre-existing immunity is unclear, but preliminary data from the STEP trial (Human Immunodeficiency Virus [HIV] vaccine MRKAd5 HIV-1gag/pol/nef in subjects at high-risk for HIV infection) suggested that HIV incidence was higher among Ad5 seropositive vaccines when compared with placebo recipients (5.1% vs. 2.2% per year).<sup>32</sup> Among those who were Ad5 seronegative, HIV incidence was similar, implying an independent effect of seropositivity on HIV acquisition. Whether this relationship holds for other adenovirus serotypes, or for other antigen inserts, is unknown but we modified our protocol to exclude volunteers with pre-existing Ad35 antibody.

The primary limitation we observed in this Phase I study was that of increasing reactogenicity with higher doses. Not

only did the overall frequency of solicited local and systemic reactions increase in the 10<sup>10</sup> and 10<sup>11</sup> vp/mL groups, but the intensity of these events was greater, with 4/16 (25%) of individuals in the highest dose group experiencing a severe systemic reaction, defined as interfering with activities of daily living. Typically these symptoms consisted of fever, headache, myalgias, and malaise. Similar reactogenicity has been observed in some other human studies of adenovirus-vectored vaccines.<sup>33</sup> In the HIV Vaccine Trials Network (HVTN) 054 study of an Ad5 HIV vaccine, doses of 10<sup>11</sup> vp/mL were associated with both a higher frequency and severity of malaise (p = 0.025), myalgia (p = 0.004), headache (p = 0.007), fever (p = 0.005) and chills (p < 0.001) when compared with the 10<sup>10</sup> vp/mL dose. However, the higher dosages were required to generate modest immune responses. Future studies will focus on strategies to improve immunogenicity, while reducing reactogenicity, such as modified dose regimens (e.g., 1 × 10<sup>10</sup> vs. 5 × 10<sup>10</sup> vp/mL) and various prime-boost strategies with other malaria vaccine candidates. In fact, the approach of heterologous prime boost is currently being evaluated with Ad35.CS.01 as priming vector and Ad26.CS.01 as booster in human challenge study as well as RTS, S as booster in human challenge study (NCT01397227 and 01366534, <http://www.clinicaltrials.gov>).

As the first administration of the Ad35.CS.01 vaccine in humans, this study has clear implications in the development of a successful immunization strategy against malaria in at-risk hosts. There are certain limitations that should be discussed, however. While 72 subjects is robust for a Phase I study, the sample size is not large enough to evaluate small differences in immunogenicity or reactogenicity that may be influenced by age, gender, race/ethnicity, or other variables. Further, our inability to interpret

**Table 2.** Geometric mean titers of CS antibodies at baseline, 1 mo, 2 mo, 6 mo, and 7 mo following first vaccination

Dosage group	Geometric mean titer (95% confidence interval)				
	Baseline	Day 28*	Day 56 <sup>†</sup>	Day 180 <sup>‡</sup>	Day 208 <sup>§</sup>
10 <sup>8</sup> vp/mL	9.0	9.0	9.0	9.0	9.7 (8.2, 11.5)
10 <sup>9</sup> vp/mL	9.4 (8.5, 10.4)	10.3 (8.4, 12.7)	22.4 (10.7, 47.0) <sup>b</sup>	13.7 (8.3, 22.8)	17.1 (9.2, 31.5) <sup>a</sup>
10 <sup>10</sup> vp/mL	9.0	14.1 (10.0, 19.9) <sup>a</sup>	34.4 (21.1, 56.2) <sup>c</sup>	10.9 (8.7, 13.6)	20.8 (11.2, 38.6) <sup>b</sup>
10 <sup>11</sup> vp/mL	9.0	26.5 (16.5, 42.4) <sup>c</sup>	52.0 (26.1, 103.7) <sup>c</sup>	19.9 (9.6, 41.1) <sup>a</sup>	61.2 (21.3, 175.9) <sup>c</sup>
Placebo	9.0	9.0	9.0	9.0	9.0

A 4-fold rise in antibody titers from baseline was seen only among the 10<sup>11</sup> vp/mL dose group. \*1 mo post first vaccination; <sup>†</sup>1 mo post second vaccination; <sup>‡</sup>Prior to third vaccination (6 mo post first vaccination); <sup>§</sup>1 mo post third vaccination. Negative responses = 9.0 Elisa units, calculated as half the lower limit of quantification; <sup>a</sup>p < 0.05; <sup>b</sup>p < 0.01; <sup>c</sup>p < 0.001 for comparison of mean log Elisa units vs. placebo.

epitope-specific T-cell responses due to high baseline ELISpot values limits our ability to evaluate this important aspect of the immune response; nevertheless, the antibody responses are reassuring and provide guidance on the dosing that will be required in subsequent studies.

In this population of malaria naïve adults, Ad35.CS.01 was immunogenic and induced antibody responses against the CS protein in a dose dependent manner. Priming was successfully achieved in most of the subjects receiving the dose of 10<sup>10</sup> and 10<sup>11</sup> vp/mL, with the highest titers observed in the 10<sup>11</sup> vp/mL dose group. Our study demonstrates the overall safety of a novel vaccine candidate for the potential prevention of malaria that induces humoral immune responses in the higher dose groups. Ongoing challenge studies of Ad35.CS.01 seek to assess its efficacy.

## Materials and Methods

### Trial design

The study was a randomized, placebo-controlled clinical trial to assess the safety of four ascending dosages of the Ad35 circumsporozoite-protein containing malaria vaccine administered in 3 intramuscular doses given on a 0, 1, and 6 mo schedule. Subjects were allocated to vaccine or placebo in a 5:1 ratio within each sequentially enrolled dosage group. Dose escalation proceeded following Safety Monitoring Committee (SMC) safety review 14 d after the first vaccination in the prior dosage group. The study, conducted at Vanderbilt University Medical Center and Stanford University Hospital, was approved by the Institutional Review Boards of each institution and registered as clinical trial identifier NCT00371189.

### Study population

Subjects were included in the study if they were between the ages of 18 and 45 y; were in good health, as determined by screening medical history, physical examination, and laboratory assessments; and were able to comply with protocol requirements. Subjects were excluded for the following conditions: currently receiving corticosteroids or other immunosuppressive agents; abnormal screening laboratory values as outlined in Study Schedule below; detectable neutralizing antibody titers against Ad35 prior to enrollment; history of intravenous drug or alcohol abuse; recent receipt of live vaccines (30 d), subunit vaccines

(14 d) or blood products (6 mo); history of malignancy; or positive serology for HIV, hepatitis C virus (HCV), or hepatitis B virus (HBV). Subjects were also excluded for a past history of malaria infection or vaccination, travel to a malaria endemic area within the previous 12 mo, or anticipated travel to a malaria-endemic area prior to conclusion of the trial (Day 208).

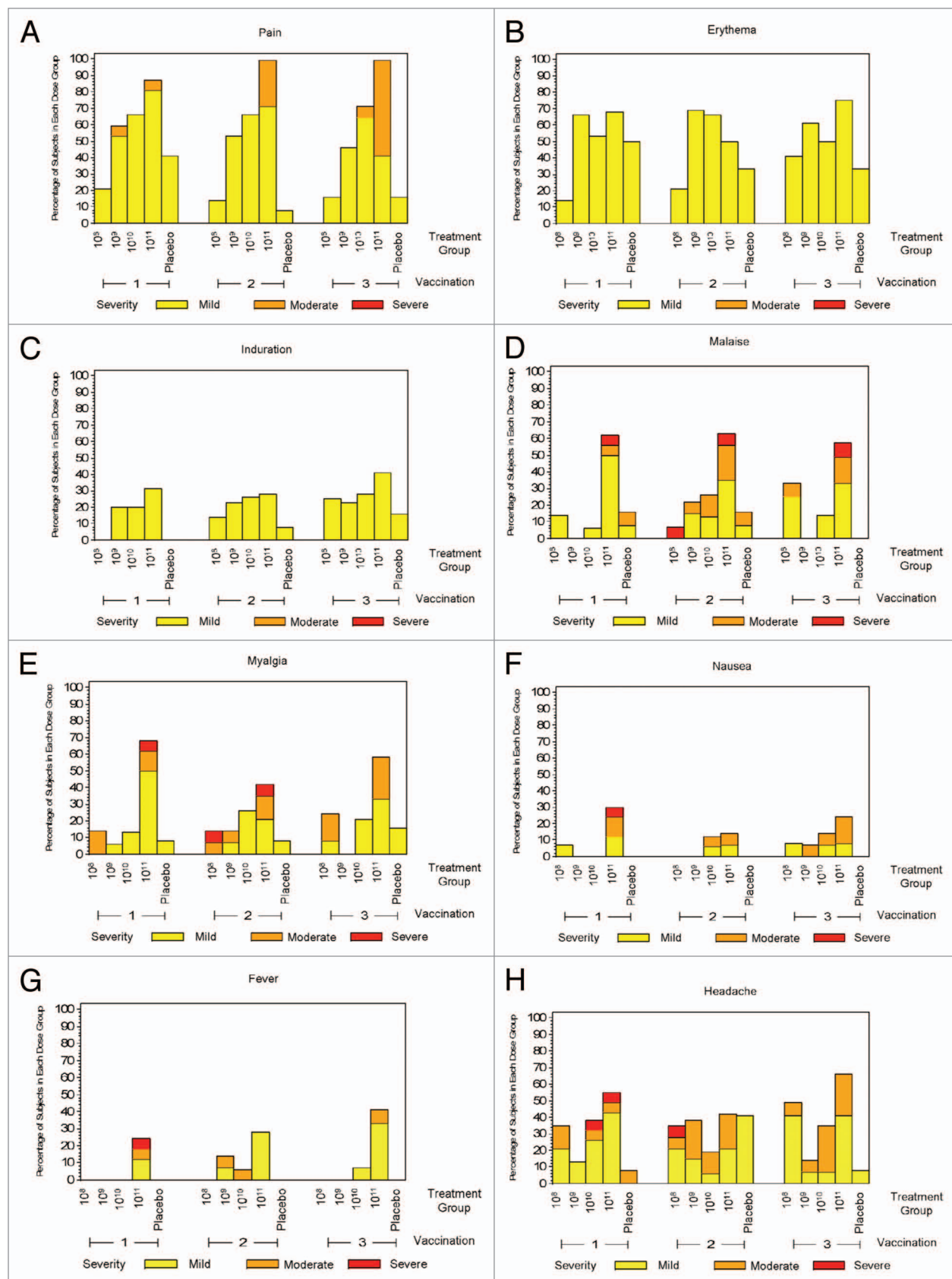
### Vaccine

Ad35.CS.01 is a non-adjuvanted malaria vaccine with a codon optimized nucleotide sequence representing the *P. falciparum* circumsporozoite (CS) surface antigen inserted into a replication deficient Adenovirus 35 backbone. It was produced in complementing PER.C6<sup>®</sup> cells by Crucell Holland BV, Leiden, The Netherlands, under National Institutes of Health (NIH), Division of Microbiology and Infectious Diseases (DMID) supported contract NOI-AIO5421. The *P. falciparum* CS gene insert is a synthetic, mammalian-codon-optimized insert encoding a CS protein as previously described.<sup>34</sup> Normal saline was used for placebo administration. The vaccine doses were 10<sup>8</sup> vp/mL, 10<sup>9</sup> vp/mL, 10<sup>10</sup> vp/mL, and 10<sup>11</sup> vp/mL. The concentration of the vaccine was determined by optical density (OD<sub>260</sub>). The absorbance at OD<sub>260</sub>, after formulation buffer background subtraction, was multiplied by the Maizel factor to attain the number of virus particles.<sup>35</sup> Each measurement is calibrated against the reference, which should fall within pre-determined control limits. The strength of the product (i.e., the ratio of virus particles to infectious units) was determined by the vp/IU ratio obtained from the OD<sub>260</sub> and the tissue culture infective dose (TCID<sub>50</sub>) assay. The vp/IU ratio was below 30:1, confirming release criteria.

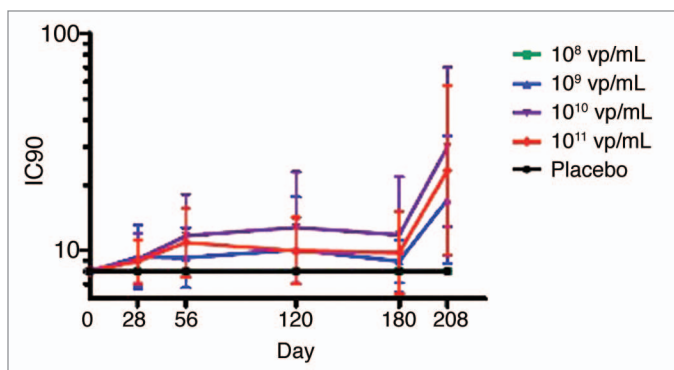
Three doses of either vaccine or placebo were administered on a 0, 1, and 6-mo schedule. Each 1.0 mL dose of the assigned dose of Ad35.CS.01 or placebo was administered using a sterile, disposable syringe and needle by intramuscular injection into the deltoid muscle by an unblinded vaccinator with no further communication with the subject regarding adverse events. Subsequent injections were administered in alternating arms.

### Study schedule

Subjects were evaluated prior to vaccination with a medical history, determination of concomitant medications, physical examination and screening laboratory studies that included: hematology (hemoglobin, white blood cell count [WBC], absolute neutrophil count [ANC], and platelet count); chemistries



**Figure 3.** Reactogenicity following each dose of Ad35.CS.01 vaccine at increasing doses. Local and systemic reactions appeared to increase with ascending dosages, but not with multiple administrations. (A) Pain; (B) Erythema; (C) Induration; (D) Malaise; (E) Myalgia; (F) Nausea; (G) Fever; and (H) Headache



**Figure 4.** Geometric mean Ad35 Neutralizing antibody titers at baseline, 1 mo, 2 mo, 6 mo, and 7 mo following first vaccination. Negative responses are set at 8 IC<sub>90</sub>, calculated as half the lower limit of quantification, whiskers indicate 95% CI.

(glucose, sodium, potassium, alanine aminotransferase [ALT], aspartate aminotransferase [AST], and creatinine); serology (HIV, HCV, Hepatitis B Surface Antigen [HBsAg]); and pregnancy testing (if applicable). In addition, a urine sample was obtained for blood and protein detection and for adenovirus culture. Due to preliminary results from the HIV-STEP Trial,<sup>32,36</sup> subjects in dose cohorts 3 and 4 (10<sup>10</sup> and 10<sup>11</sup> vp/mL, respectively) were screened for Ad35 serum antibodies prior to enrollment<sup>21</sup> and excluded from enrollment if seropositive. HIV antibody testing was performed at screening and at one year after the first vaccination.

#### Outcomes

The primary objective of the study was to assess the safety and reactogenicity of ascending dosages of the vaccine through laboratory studies and daily reactogenicity diaries (Fig. 4). Subjects were asked to rate both local symptoms (arm pain, bruising, presence of erythema, and presence of induration) and systemic symptoms (fever, malaise, myalgia, headache, chills, nausea, and vomiting) for 14 d after each vaccination. Severity grades were mild (symptoms present), moderate (symptoms interfere with some activities), or severe (symptoms interfere with activities of daily living). These symptoms were reviewed on Day 1 after vaccination (telephone call), and on Days 2, 7, and 14 after vaccination with clinic visits. Study personnel verified the severity rating and study physicians assigned association with the study product. In addition, one week after each vaccine dose, identical safety laboratory testing was performed as at enrollment. Unsolicited adverse events (AEs) were collected through 28 d after the last dose and serious adverse events (SAEs) were collected until trial completion. Secondary objectives included humoral immunogenicity at various time points after each vaccination. Assessment of cellular immunity was included as an exploratory endpoint.

#### Serologic assays

Sera were collected at baseline, 1 mo after the 1st and 2nd vaccination, 5 mo after the 2nd vaccination (i.e., immediately prior to the 3rd vaccination) and 1 mo after the 3rd vaccination. ELISA methodology was used at Crucell, as previously described, to determine IgG antibody concentrations to CS protein using a (NANP)<sub>6</sub> peptide as capture antigen.<sup>37</sup> In short, the peptide

was obtained from Pepscan (Lelystad, Netherlands) at a purity of 90%. The peptide was coated at a concentration of 2 µg/ml in 0.05M carbonate buffer at 2–8 °C overnight or for a maximum of 3 d. Reference sera, serum samples, and internal controls were diluted in phosphate-buffered saline (PBS), 2% gelatin, and 1% human serum (dilution buffer) and incubated for 1 h at room temperature. Caprine anti-human IgG horseradish peroxidase was added and incubated for 1 h at room temperature. Finally, *o*-phenylenediamine (OPD; Sigma Aldrich) was added for the colorimetric reaction, which was stopped after 10 min using 5.3% H<sub>2</sub>SO<sub>4</sub> stop solution. Subsequently, the optical density (OD) was measured at 492 nm, using a Bio-Tek microplate spectrophotometer PowerWave 340. The titer is expressed in relative ELISA Units/mL (EU/mL), by assessing the titer of a sample in the linear portion of a standard curve run in parallel with a nominal binding activity. The limit of detection for the anti-CS assay was 18 EU/mL. Values below this threshold were set at 50% of the limit of detection. In addition, representative sera will be available through the International Reference Center for Malaria Serology Laboratory, located at the Walter Reed Army Institute of Research.

Ad35-specific neutralizing antibody titers were assessed by luciferase-based virus neutralization assays as described previously<sup>21</sup> and recently optimized and validated for human serum. In short, sera were heat inactivated and serially diluted by 2-fold (starting dilution is 1:16). Ad35.Luc solution (10<sup>8</sup> vp/ml) was added to each well at 500 virus particles per cell. A549 cells were added at 10<sup>4</sup> cells/well and plates were incubated at 37 °C/10% CO<sub>2</sub> for 24 to 26 h. After incubation, medium was discarded, PBS was added, and plates were stored frozen overnight. Plates were allowed to thaw at room temperature, Luciferase Steady-Lite substrate was added and the lysate was transferred into black/white isoplates. Luminescence counts were recorded on a 1450 MicroBeta Trilux. Titers were determined by validated software ANAM (Adenovirus neutralization Assay Macro). The limit of detection for the Ad35 neutralizing antibody assay was 16 IC<sub>90</sub>, the titer at which 90% neutralization was achieved. Values below this threshold were set at 50% of the limit of detection.

Peripheral blood mononuclear cells were collected for assessment of cellular immune responses prior to each vaccination and one month following the 2nd and 3rd vaccinations. Characterization of antigen-specific T cell responses were evaluated based on the quantity and phenotype of cells responding to CS-antigen using an ELISpot assay as the primary quantitative readout and an IFN-γ ICS assay to assess the CD4<sup>+</sup> vs. CD8<sup>+</sup> T-cell response. These assays have been described in more detail elsewhere.<sup>38–41</sup> Due to unusually high baseline ELISpot values in multiple volunteers and overall sample degradation, these data were uninterpretable and not included in the results.

#### Sample size

Fifteen subjects per dosage group were proposed to receive the Ad35.CS.01 vaccine to obtain preliminary safety information on a small cohort of subjects before proceeding to subsequent larger trials. The study was powered to detect at least a 10% rate of all adverse events with 80% power but was not powered to compare dosage-related immune responses.



## Randomization and blinding

Subjects were randomized through the AdvantageEDC<sup>SM</sup> Data Entry System (provided by the EMMES Corporation) with a 5:1 vaccine to placebo ratio. Study staff members were blinded to assignment with the exception of the investigational pharmacist and the unblinded vaccinator.

## Statistical methods

The study was designed as an exploratory study to estimate event rates and patterns of immune responses rather than to test formal statistical hypotheses. As a result, estimates are presented with their 95% confidence intervals. Adverse events and immune responses were reported as frequencies or means. Fisher's exact tests for categorical and Kruskal-Wallis tests for continuous variables were used to compare safety and immunogenicity outcomes in the vaccine groups relative to placebo.

## Disclosure of Potential Conflicts of Interest

MGP, JH, and IV are employees of Crucell, Leiden, The Netherlands. All other authors report no relevant conflicts of interest.

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## Supplemental Materials

Supplemental materials may be found here:  
www.landesbioscience.com/journals/vaccines/article/26038

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